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Short communication

Synergistic antiviral activity of acyclovir and vidarabine against herpes simplex virus types 1 and 2 and varicella-zoster virus

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Abstract

Acyclovir and vidarabine both exhibit anti-herpetic activity. Because different mechanisms of action of vidarabine and acyclovir have been reported, we analyzed their combined anti-herpetic activity on plaque formation of herpes simplex virus (HSV)-1, HSV-2, and varicella-zoster virus (VZV) by isobolograms. The results indicate that acyclovir and vidarabine have a synergistic effect on wild type HSV-1, HSV-2, and VZV. The susceptibility of thymidine kinase-deficient HSV-1 to vidarabine was not affected by the presence of acyclovir, suggesting that phosphorylation of acyclovir is essential for synergism. The combined anti-HSV activity of acyclovir and vidarabine against phosphonoacetic acid-resistant HSV-1 with DNA polymerase mutation did not show synergism in contrast to that against wild-type herpesviruses. Alteration of the substrate specificity of viral DNA polymerase to acyclovir and vidarabine annihilated the synergism. Thus, the nature of their binding sites on DNA polymerase is important to the synergistic anti-herpesvirus activity of acyclovir and vidarabine.

Keywords: Herpes simplex viruses; Varicella-zoster virus; Drug interactions; Synergism; Acyclovir; Vidarabine

Acyclovir has antiviral activity against herpesviruses such as herpes simplex virus (HSV)-1, HSV-2, and varicella-zoster virus (VZV) (Biron and Elion, 1980; Elion et al., 1977). Acyclovir was developed in the 1970s and is the agent of first choice for herpesvirus infection due to its efficacy and safety. It is a nucleoside analogue that exhibits anti-herpetic activity after phosphorylation by viral thymidine kinase (TK). Acyclovir triphosphate interferes with viral DNA polymerization through competitive inhibition with guanosine triphosphate and obligatory chain termination (Biron and Elion, 1980; Coen and Schaffer, 1980; Elion et al., 1977; Larder and Darby, 1986). The incorporation of acyclovir–monophosphate into the template-primer followed by the binding of the next nucleotide encoded by the template resulted in the formation of a tight dead-end complex (Reardon and Spector, 1989).

Vidarabine (arabinosyladenine) was identified in an extract of a Caribbean sponge (*Cryptotethia crypta*) (de Rudder and de Garilhe, 1965; Schabel, 1968). It is a purine nucleotide analog that inhibits viral DNA synthesis (Gephart and Lerner, 1981; Shipman et al., 1976). Although vidarabine has been used for the treatment of herpesvirus infections, acyclovir is more frequently used since vidarabine has less efficacy and more toxicity than acyclovir (Collum et al., 1983; Reusser et al., 1996; Shepp et al., 1986; Shope et al., 1983; Whitley et al., 1976, 1991, 1992). Vidarabine is capable of inhibiting acyclovir-resistant/TK-deficient mutants of HSV and VZV, because it is phosphorylated to its active vidarabine–triphosphate form by cellular kinases and is not dependent for its activation on the viral TK (Kamiyama et al., 2001; Schwartz et al., 1984; Shiraki et al., 1990).

In this study, we assessed the synergistic antiviral activity of acyclovir and vidarabine on HSV-1, HSV-2, and VZV by a plaque reduction assay. We showed that the binding sites of phosphorylated acyclovir and vidarabine on the viral DNA polymerase were essential to the synergy by using TK-deficient HSV-1 and phosphonoacetic acid (PAA)-resistant HSV-1.

Acyclovir and vidarabine were purchased from Sigma, MO, and dissolved in dimethylsulfoxide at 10 mg/ml. Vero cells were grown and maintained in Eagle's minimum essential medium (MEM) supplemented with 5% and 2% heat-inactivated newborn calf serum, respectively. Human embryonic lung (HEL) cells were grown and maintained in MEM supplemented with 10 and 2% heat-inactivated fetal bovine serum (FBS),

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respectively. The HSV-1 strains used were the wild type 7401H strain (Kumano et al., 1987; Kurokawa et al., 1993; Okuda et al., 2004), PAA-resistant HSV-1 (Kurokawa et al., 1995, 2001), and TK-deficient HSV-1, B2006 strain (Dubbs and Kit, 1964; Kurokawa et al., 2001). The HSV-2 strain used was a wild type genital isolate (OOM strain) provided from Dr. T. Kawana, Teikyo University, Japan. The wild type Oka strain was used for VZV infection (Shiraki et al., 1984). The HSV stocks were prepared from infected Vero cells by freezing and thawing the infected culture, followed by centrifugation (Kurokawa et al., 1995; Okuda et al., 2004; Yoshida et al., 2005). VZV was propagated in HEL cells, and cell-free virus was prepared in SPGC medium (phosphate buffered saline containing 0.1% sodium glutamate, 5% sucrose, and 10% FBS) as described previously (Shiraki et al., 1982, 1984, 1990, 2003).

To evaluate the antiviral efficacy of acyclovir and vidarabine on HSV or VZV infection, a standard plaque reduction assay was performed as described previously (Miwa et al., 2005; Okuda et al., 2004; Yoshida et al., 2005). Confluent Vero cells and HEL cells in 60 mm dishes were infected with 100 plaque-forming units/0.2 ml of HSV and VZV, respectively, for 1 h. The 1% methylcellulose nutrient culture medium were prepared just before use by adjusting the concentrations of acyclovir alone (0, 0.1, 0.2, 0.5, 1, 2, 5, or 10 μg/ml), vidarabine alone (0, 1, 2, 5, 10, 20, or 30 μg/ml), or a mixture of acyclovir and vidarabine at various concentrations as stated above and overlaid on the infected cells. The cells were incubated at 37 °C for 3 and 5 days for HSV and VZV, respectively, and fixed with 5% neutral formalin followed by staining with 0.03% methylene blue. The number of plaques was counted under a dissecting microscope. The 50% inhibitory concentration for plaque formation (IC_{50}) was defined as the concentration at which the plaque number decreased to half of that in cells cultured without the addition of antiviral drugs. The IC_{50} was determined by using the computer program Microplate Manager III (BioRad, Hercules, CA).

In order to analyze the interaction of acyclovir and vidarabine graphically, the IC_{50}s of these agents in their various concentrations were plotted as an isobologram (Kurokawa et al., 2001). Synergy and antagonism are defined as deviations from dose-wise additivity, which results when two drugs interact as if they were the same drug. Curves falling below the line of additivity indicate synergy, curves on the line indicate an additive reaction, and curves above the line indicate an antagonistic reaction.

Vero cells were seeded at a concentration of 1.0×10^5 cells/well in 12-well plates and were grown at 37 °C for 3 days. The culture medium was replaced with fresh medium containing 10 μg/ml of acyclovir alone, 30 μg/ml of vidarabine alone, and their combination for the cytotoxicity assay. The cells were cultured for 3 days and the number of viable cells was determined by the Trypan blue-exclusion test.

The ratio of viable cells treated with 10 μg/ml of acyclovir, 30 μg/ml of vidarabine, and their combination was 99.8%, 107.6%, and 107.5% of control cells containing 2.0×10^5 cells/well, respectively. Thus, acyclovir and vidarabine did not show cytotoxicity to Vero cells under the experimental conditions used.

Table 1
The IC_{50}s of acyclovir and vidarabine against HSV-1, HSV-2, PAA-resistant HSV-1, and TK-deficient HSV-1

Viruses	$IC_{50} \pm$ S.E. μg/ml (μM)	
	Acyclovir	Vidarabine
HSV-1	0.85 ± 0.15 (3.78 ± 0.58)	9.3 ± 0.7 (34.7 ± 2.4)
HSV-2	0.86 ± 0.06 (3.83 ± 0.33)	11.3 ± 0.6 (42.1 ± 1.8)
PAA-resistant HSV-1	4.38 ± 1.1 (19.4 ± 4.9)	21.1 ± 2.3 (78.9 ± 8.7)
TK-deficient HSV-1	>10 (44.4)	14.1 ± 2.0 (62.7 ± 8.6)

The antiviral efficacy of acyclovir and vidarabine on HSV was examined by the plaque reduction assay. HSV-infected vero cells were treated with the drugs at concentrations of 0, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, or 30 μg/ml. The IC_{50}s are expressed as the mean (μg/ml) ± S.E. of four to six independent experiments. The figures in parentheses are the IC_{50}s expressed as (μM). The PAA-resistant strain was significantly resistant to acyclovir ($P < 0.02$) and vidarabine ($P < 0.02$), compared to HSV-1 and HSV-2 by the Student's *t*-test.

The susceptibilities of viruses to acyclovir and vidarabine were determined by the plaque reduction assay (Table 1). The IC_{50}s of acyclovir were similar in wild type HSV-1 and HSV-2, but the PAA-resistant HSV-1 had about five-fold less susceptibility to acyclovir than wild-type HSV-1. The IC_{50}s of vidarabine were similar for HSV-1 and HSV-2, but those of both PAA-resistant HSV-1 and the TK-deficient HSV-1 were about 1.5–2-fold more than that of wild type HSV-1. The PAA-resistant HSV-1 strain showed resistance to both acyclovir and vidarabine compared with wild-type HSV-1 and HSV-2.

The interaction of acyclovir and vidarabine on the plaque formation of HSV-1, HSV-2, and VZV is shown in Fig. 1A–C. The curve fell below the line of the additive effect at all concentrations in HSV-1, HSV-2, and VZV, indicating that the combination of acyclovir and vidarabine exhibited synergism against three viruses. Thus, synergisms of acyclovir and vidarabine were consistently observed in wild-type HSV-1, HSV-2, and VZV.

To elucidate the mechanism of this synergism, we next investigated the effect of apparent lack of phosphorylation of acyclovir on the synergism by using a TK-deficient HSV-1 that does not phosphorylate acyclovir. There was no significant change in the IC_{50} value of TK-deficient HSV-1 to vidarabine in the presence and absence of acyclovir (Table 2). Thus, phosphorylation of acyclovir by TK was needed to modify the susceptibility to vidarabine, indicating the importance of phosphorylated acyclovir for synergism with vidarabine.

Secondly, PAA-resistant HSV-1, which has a DNA polymerase alteration, was used to analyze the interaction of phosphorylated acyclovir and vidarabine on DNA polymerase. Fig. 1D shows the isobologram of acyclovir and vidarabine for PAA-resistant HSV-1, and the plotted line overlapped with the additive line. The combination failed to show synergy, but the additive effect of the drug combination against PAA-resistant HSV-1 remained. Thus, alteration of the viral DNA polymerase structure abolished the synergistic action of acyclovir and vidarabine on HSV. This indicated that the phosphorylated forms of acyclovir and vidarabine worked synergistically on the DNA polymerase of wild type herpesviruses.

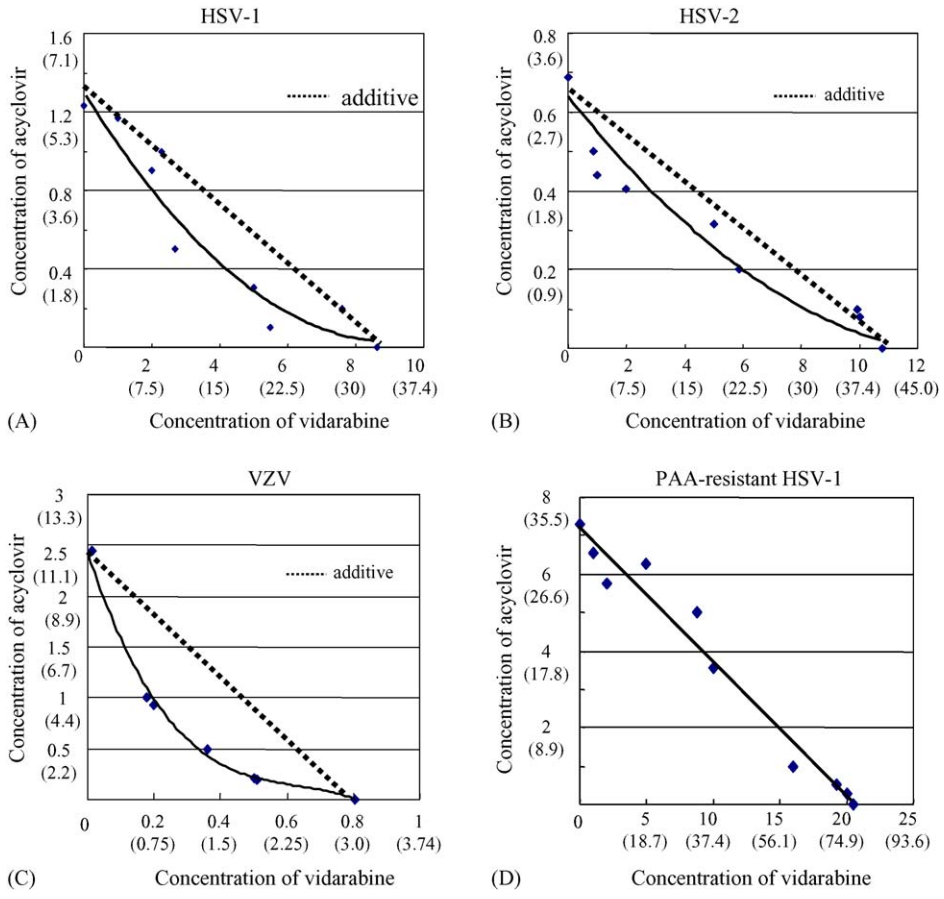

Fig. 1. Interaction of acyclovir and vidarabine on wild type HSV-1, HSV-2, and VZV plaque formation analyzed by isobolograms. The figure is one of three independent experiments using HSV-1 (A), HSV-2 (B), VZV (C), and PAA-resistant HSV-1 (D). The dotted lines indicate the theoretical additive activity. The measured lines were significantly lower than these dotted lines in (A–C) ($P < 0.02$, $P < 0.02$, $P < 0.001$, respectively) by the paired t-test, indicating synergy. In (D), the plotted line coincided with the additivity line, indicating the additive anti-HSV activity for the combination of acyclovir with vidarabine against PAA-resistant HSV-1. The drug concentrations are expressed as μg/ml (μM).

We previously reported the differences in activities between acyclovir and vidarabine treatment in VZV infection in vitro and showed that the antiviral status induced by vidarabine continues longer than that induced by acyclovir (Miwa et al., 2005). The proposed mechanisms of antiviral activity of vidarabine are (i) inhibition of the viral DNA polymerase by vidarabine triphosphate due to competitive inhibition of deoxyadenosine triphosphate (Muller et al., 1977b), (ii) inhibition of virus-induced ribonucleotide reductase by either vidarabine triphosphate or vidarabine diphosphate (Cohen, 1972; Langelier and Buttin, 1981), and (iii) selective incorporation of vidarabine monophosphate into viral DNA causing a decrease in the rate of primer elongation and chain termination (Pelling et al., 1981; Muller et al., 1977a).

Acyclovir-resistant DNA polymerase mutants of VZV can be divided in two groups, one that has less susceptibility to both vidarabine and PAA and one that is hypersensitive to both vidarabine and PAA (Kamiyama et al., 2001).

In this study, we showed the synergism of acyclovir and vidarabine against wild type HSV-1, HSV-2, and VZV infection. The importance of phosphorylated acyclovir for synergism with vidarabine was shown by the analysis of TK-deficient virus. Because the alteration of herpesviral DNA polymerase causes a change of substrate specificity for both acyclovir and vidarabine (Kamiyama et al., 2001), the change of DNA polymerase activity may have abolished their synergism. This indicates that the triphosphate forms of both acyclovir and vidarabine are essential for their synergism and that the nature of their binding sites on DNA polymerase is important for their synergistic antiviral effects against herpesviruses.

Table 2
The IC_{50}s of vidarabine against TK-deficient HSV-1 in the presence and absence of acyclovir

Acyclovir	Vidarabine $IC_{50} \pm$ S.E. μg/ml (μM)
0 (0)	14.1 ± 2.0 (62.7 ± 8.6)
5 (22.2)	14.7 ± 1.3 (65.4 ± 5.9)
10 (44.4)	14.5 ± 2.2 (64.6 ± 10.0)

The antiviral efficacy of vidarabine on TK-deficient HSV-1 in the presence and absence of acyclovir was examined by plaque reduction assay. TK-deficient HSV-1-infected vero cells were treated with vidarabine at concentrations of 0, 1, 2, 5, 10, 20, or 30 μg/ml in combinations with 0, 5, or 10 μg/ml of acyclovir. The number of plaques was counted under a dissecting microscope and the IC_{50} was determined. The IC_{50}s are expressed as the mean (μg/ml) ± S.E. of three to five independent experiments. The figures in parentheses are the IC_{50}s expressed as (μM).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2006.05.001.

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